REMARKS

Reconsideration of the present application is respectfully requested. Claims 31-39 are pending.

Claims 1-21 have been cancelled without prejudice as belonging to a non-elected invention. Applicant affirms the election of Group I, claims 1-18, and 21 and the election of SEQ ID NOS: 3 and 4.

Claims 29, 30, 37, 38, and 39 have been cancelled without prejudice or disclaimer. Applicants expressly reserve the right to file divisional applications or take such other appropriate measures deemed necessary to protect the subject matter of cancelled claims.

Claims 23, 24, 28, 31, 32, 33, 34, 35, and 36 have been amended to reflect the elected invention and incorporate the Examiner's suggestions.

The specification has been amended to address the Examiner's objections.

No new matter has been added by way of amendment to the claims and specification.

The marked up version of the claim and specification amendments is found on a separate sheet attached to this amendment and titled **"Version with Markings to Show Changes Made."** In showing the changes, deleted material is shown in brackets and inserted material is shown as underlined. It is respectfully requested that the amendments be entered.

Specification

The disclosure is objected to for containing embedded hyperlinks on pages 6, 32, 40, and 44. The specification has been amended to delete the hyperlinks.

The specification is objected to for disclosing sequences that are not properly identified by sequence identifiers. Such sequences occur on pages 33, 39, 40, 43, and 55. The specification and Sequence Listing have been amended to reflect sequence identifiers where necessary.

Oath/Declaration

Applicant is notified that the Oath or Declaration is defective due to non-initialed and/or non-dated alterations. A substitute Oath or Declaration is being submitted in compliance with 37 CFR 1.67(a).

Information Disclosure Statement

The Examiner states: "The a [sic] number of citations listed on the information disclosure statements filed 6/14/00, 6/23/00, and 11/6/00 fail to comply with the provisions of 37 CFR 1.97, 1.98 and MPEP §609 because the citations of references that are identified by title and 'accession numbers' are not complete. These citations do not provide the name of the database whose accession numbers are represented."

It is believed the Information Disclosure Statements are fully compliant as filed. Applicant can find no recitation in 37 CFR 1.97, 1.98 or MPEP §609 that states the name of the database whose accession numbers are represented is a required notation in the IDS. Applicant has provided the name of submitter, year, accession number and source species of each reference. This information is sufficient for any individual to readily retrieve the sequence of interest. Examiner is requested to cite the precise language that requires applicant to accept the burden and expense of re-submitting the disclosure statements to include the database name.

Rejections under 35 U.S.C. §112

Rejections under 35 USC §112, second paragraph

Claims 22-39 are rejected under §112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

The Examiner states: "Claims 22-39 are rejected over the recitation of 'phytyl/prenyltransferase protein' and "phytyl/prenyltransferase polynucleotide'. The

specification does not provide a clear definition which identifies these proteins, and thus it is not possible to determine the meets [sic] and bounds of these claims".

The rejection is respectfully traversed. Page 4, lines 5-8, of the specification reads: "The present polypeptides catalyze the condensation of homogentisic acid with phytyldiphosphate or geranylgeranyl pyrophosphate to produce the first intermediates in tocopherol or tocotrienol synthesis, respectively." It is believed one of skill in the art would be properly notified by the above language of the metes and bounds of claims 22-39.

Claims 22, 27, and 29 are rejected over the recitation of method steps for "growing the plant cell". The Examiner states: "These are unclear because growing the cell will not result in a plant, rather the method step should be directed to regenerating."

The rejection is respectfully traversed. The entire phrase of the claims reads: "... growing the plant cell under plant growing conditions to produce a *regenerated* plant ...." The claim currently is directed to regenerating. It is believed one of skill in the art would readily understand the phrasing of the current claim.

The Examiner also states: "Claims 22, 27 and 29 further recited 'capable of expressing' which is indefinite because it is unclear whether or not the polynucleotide is expressed. The phrase should be changed to which expresses."

Claims 22 and 27 have been amended to incorporate the Examiner's suggestion. Claim 29 has been cancelled without prejudice. The amendment to the claims obviates the rejection.

The Examiner states: "In claim 24, the recitation of 'vegetables, peppers, potatoes, apples' (plural) is inconsistent with 'plant' (singular) in line 1 of the claim.

The claim has been amended to make the recitation of species consistent with the singular "plant".

Rejections under 35 USC §112, first paragraph

Claims 31-39 are rejected under 35 USC §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

The Examiner states: "Claims 31-39 are directed to methods for modulating the level of a phytyl/prenyltransferase protein in a plant which comprise transforming a plant cell with a phytyl/prenyltransferase polynucleotide and growing the plant cell under conditions to produce regenerated plant, wherein the phytyl/prenyltransferase is selected from polynucleotides having at least 70% sequence identity to SEQ ID NO: 3, and polynucleotides which selectively hybridize to SEQ ID NO: 3 ... the specification only describes a singe protein and a single cDNA encoding that protein and fails to teach or describe any other polynucleotides that are related to SEQ ID NO: 3 within the limitations of the rejected claims."

Claims 37, 38, and 39 have been cancelled without prejudice.

The Examiner asserts that the specification only provides guidance for a single protein and a single cDNA encoding that protein and does not adequately describe the broadly claimed genus that encompasses "any other polynucleotides that are related to SEQ ID NO: 3 within the limitations of the rejected claims".

It is well within the ability of one of skill in the art to produce polynucleotide sequences within the limitations of the claims. Such guidance is given in the specification beginning on page 8, the section entitled "NUCLEIC ACIDS". Methods for using sequence alignment are given beginning on page 5, line 31.

The Examiner is reminded that every species encompassed by the claims need not be disclosed in the specification to satisfy the written description requirement. *Utter v. Hiraga*, 845 F.2d 993,6 USPQ2d 1709 (Fed. Cir. 1988).

The Examiner further asserts: "The specification provides no guidance as to how or where the disclosed polynucleotide can be modified yet still maintain the functionality required for the instant methods. The claims also fail to recite other

relevant identifying characteristics (physical and/or chemical and/or functional characteristics coupled with a known or disclosed correlation between function and structure).... Therefore there is a lack of guidance or teaching regarding structure and function because there is only a single example provided in the specification and because there is no guidance found in the instant specification."

The rejection is respectfully traversed.

The Federal Register (Vol. 66, No. 4, Jan. 5, 2001, page1106, column 3, third paragraph) recites: "For each claim drawn to a genus: The written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species by actual reduction to practice ... reduction to drawings ... *or by disclosure of relevant, identifying characteristics, i.e., structure or other physical and/or chemical properties, by functional characteristic coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics sufficient to show the applicant was in possession of the claimed genus.*"

The application discloses structure via a DNA sequence (SEQ ID NO: 3) of an isolated polynucleotide whose overexpression increases the amount of tocopherol relative to oil in a plant tissue (see the specification, Examples: page 59, lines 26-29). The application discloses the function correlated with this structure on page 4, lines 5-8, of the specification which reads: "The present polypeptides catalyze the condensation of homogentisic acid with phytyldiphosphate or geranylgeranyl pyrophosphate to produce the first intermediates in tocopherol or tocotrienol synthesis, respectively."

Physical and chemical properties associated with the sequences utilized in the methods are defined by hybridization conditions to the disclosed sequence beginning on page 12, line 30 of the specification and structural variants are described and defined in the specification on page 9, lines 7-21 such that the skilled artisan could readily visualize that the applicant was in possession of the invention claimed.

The Examiner cites *Fiers v. Sugano* in support of the rejection. However, the Examiner has improperly applied this case to the present invention. The decision in *Fiers* turned on the conclusion that the patent by Fiers lacked a complete DNA sequence at the time of Sugano's filing which included a complete DNA sequence and a method for isolating that DNA. Note that the present application contains both a complete DNA sequence and methods for isolating that sequence (the specification beginning on page 42).

The Examiner states: "In the application at the time of filing, there is no record or description which would demonstrate conception ... of phytyl/prenyltransferase polynucleotide which has nucleotides modified by addition, insertion, deletion substitution, or inversion ...".

Examiner's attention is drawn to the specification, page 9, lines 7-13 which recites: "Variants included in the invention may contain individual substitutions, deletions or additions to the nucleic acid or polypeptide sequences. Such changes will alter, add or delete a single amino acid or a small percentage of amino acids in the encoded sequence". Clearly there was conception at the time of filing for modified phytyl/prenyltransferase polynucleotides.

The test for sufficiency of support in a parent application is whether the disclosure of the application relied upon "*reasonably* conveys to the artisan that the inventor had possession at that time of the later claimed subject matter." (MPEP 2163.02)

By disclosing the foregoing identifying characteristics, it is believed that one of skill in the art would reasonably conclude that the applicant was in possession of the claimed invention. Applicant submits that the written description requirement for claims 31-36 is satisfied.

Claims 22-39 are rejected under 35 U.S.C. §112, first paragraph, because the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with the claims.

The Examiner asserts: "The specification is enabling only for methods for modulating phytyl/prenyltransferase and tocopherols proteins in a plant which comprise transforming the plant with the isolated Synechocystis DNA, SEQ ID NO: 9, does not reasonably provide enablement for methods which utilize other polynucleotides or methods for the modulation of plastoquinone."

Claims 29, 30, and 37-39 have been cancelled without prejudice.

The Examiner appears to assert that the present specification does not provide sufficient guidance to use the elected sequence, SEQ ID NO: 3. This assumption is incorrect. Phytyl/prenyltransferase-type polypeptides were known in the art prior to the present invention and conserved regions well characterized (see Lopez, 1996, pg 3370, Fig. 2, and 3371, Fig. 4). The maize sequence used in the presently claimed methods was identified by homology to known phytyl/prenyltransferases (see the specification page 44, lines 7-23 and page 46, lines 6-12). Further, experimental data supports the conclusion that SEQ ID NO: 3 functions as a phytyl/prenyltransferase by its ability to modulate tocopherol levels in soybean somatic embryos (see the specification, beginning on page 55; and Table on page 60).

The Examiner further states: "These claims are drawn to methods for modulating the level of a phytyl/prenyltransferase protein in a plant, methods for modulating the level of tocopherol in a plant, and methods for modulating the level of plastoquinone in a plant, ... The specification demonstrates one polynucleotide which encodes a polypeptide that demonstrates phenyltransfersase activity which results in the modulation of the level of tocopherols in cells. Applicant specifically teaches that the disruption of this gene did not demonstrate any effect on the activity of plastoquinones, suggesting that there is more than one prenyltransferase active in these cells ... applicant does not teach other genes which definitively encode a phytyl/prenyltransferase."

Claims 29, 30, and 37-39 have been cancelled without prejudice.

The specification teaches sequences with demonstrated phytyl/prenyltransferase function (SEQ ID NOS: 3 and 9), and cites art that teaches prenyltransferases from seven organisms (Lopez et al.). Consequently, one of skill in the art would be well apprised of how to make and use the presently claimed methods.

The Examiner continues: "Furthermore, the specification does not provide any methods which modulate the phytyl/prenyltransferase activity such that a decrease in activity is observed."

Methods for decreasing enzymatic activity are well known in the art. The specification provides guidance in this respect on page 23, lines 2-5 by citing the relevant art. The Examiner is reminded that: "[A] patent need not teach, and preferably omits, what is well known in the art." *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 231 USPQ 81 (Fed. Cir. 1986).

It is submitted that the mere absence of evidence is insufficient to establish a case of non-enablement: "Without a reason to doubt the truth of the statements made in the patent application, the application must be considered enabling. Accordingly, the case law makes clear that properly reasoned and supported statements explaining any failure to comply with Section 112 are a requirement to support a rejection. "*In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993).

Finally, the Examiner's attention is drawn to Table 6, on page 61 of the specification. The Table shows variation of tocopherol:oil ratios in individual embryos transformed with SEQ ID NO 3. Embryos SC5 18-1 and SC5 18-2 show ratios *decreased* from the normal range of 2-5 ngT/µg oil. The effects of co-suppression or transgene silencing was well known in the art at the time the application was filed (see Montgomery et al., *Trends in Genetics*. 1998, 14(7):255-258; copy attached). One of skill in the art would recognize that co-suppression events could also occur in transformed organisms and if desired, selected for.

The Examiner further contends: "The data provided is not sufficient to support the conclusion that SEQ ID NO: 3 is a phytyl/prenyltransferase polynucleotide at all, let alone to support a conclusion that the transformation of a plant would modulate the level of a phytyl/prenyltransferase protein, a tocopherol, or a plastoquinone."

The Examiner provides an interpretation at odds with the data presented in the working examples. The Examiner states: "... data is provided from only two controls, one of whose ratios is 7.28 and the other whose ratio is 4.58 (Table 6). Thus, of the two controls, the tocopherol/oil ratio in one is above the "normal" range suggested by the applicant, and the other is at the high end of the "normal" range."

The polynucleotide sequence represented by SEQ ID NO: 3 was expressed in soybean somatic embryos as described in the example beginning on page 55, line 13. Embryos testing positive for the presence of the transgene (screened by hygromycin) were analyzed for oil and tocopherol content. (see specification, page 58, beginning on line 29). Of 33 transformed lines, 13 showed tocopherol levels increased above the normal ratios of 2-5 ngT/µg oil, as shown in Table 5, page 60. Table 6 on page 61 provides further analysis of *individual embryos* from four lines to verify the pooled data of Table 5. The "controls" (samples 15 and 18) cited by the Examiner are only controls with respect to samples 16 and 17 ... samples 15 and 18 are embryos containing the transgene and thus it would not be unexpected for these to be at the high end of the normal range.

The Examiner continues: "Applicant has characterized and isolated a single phytyl/prenyltransferase polynucleotide, namely the SLR1736 gene from Synechocystis. Applicant has not demonstrated that any of the other isolated genes have the activity of a phytyl/prenyltransferase polynucleotide. It is not clear how the other isolated genes are structurally related to the SLR 1736 gene.... Thus, it is not clear that methods utilizing these genes would accomplish the goals set out in the preamble of these claims."

It is assumed that by "other isolated genes" the Examiner is referring solely to the maize phytyl/prenyltransferase polynucleotide represented by SEQ ID NO: 3.

The other sequences belong to non-elected claims and thus are not being argued herein.

It is believed that the phytyl/prenyltransferase function of SEQ ID NO: 3 is fully supported by it's homology to known phytyl/prenyltransferase polynucleotides, the presence of conserved regions, and it's demonstrated ability to increase tocopherol levels in transformed plant tissue.

It is well established that the burden is on the Office to present arguments or evidence that the disclosure is non-enabling. The Examiner is invited to present such arguments or evidence that, despite the information contained in the disclosure, SEQ ID NO: 3 does not have the function attributed to it.

The Examiner asserts: "It is noted that the prior art provides methods which comprise transforming plant cells with nucleic acids encoding geranylgeranyl pyrophosphate (GGPP) synthase which is a phytyl/prenyltransferase protein (Ausich et al.). These plants are also considered to be within the scope of the claimed invention by virtue of their presence in the prior art."

The art is quite clear that geranylgeranyl pyrophosphate (GGPP) synthase is an enzyme which can *react with* phytyl/prenyltransferase - not a *homolog to* phytyl/prenyltransferase (for example, see Lopez, page 3369, the abstract, which reads: "The BchG gene [a phytyl/prenyltransferase] product catalyzes esterification of bacteriochlorophyllide *a* by geranylgeraniol-PP_i during bacteriocholorophyll *a* biosynthesis".) In fact, in Ausich et al, the GGPP synthase is disclosed as catalyzing the condensation of farnesyl pyrophophate (EPP) and isopentynyl pyrophosphate (IPP) to form geranyl geranyl pyrophosphate (GGPP). See column 10, second paragraph.

The Examiner further states: "... it is noted that the instant claims encompass methods which utilize nucleic acids that are related to SEQ ID NO: 3 based on hybridization or homology. However, Applicant provides no guidance for the regions of the disclosed SLR1736 gene which are essential or sufficient to encode phytyl/prenyltransferase, or for the regions of SEQ ID NO: 3 which are essential or

sufficient to encode a phytyl/prenyltransferase. In the absence of such guidance, undue trial and error experimentation would be required to screen the vast number of different polynucleotides with 70% homology to, or that would hybridize to SEQ ID NO: 3 to identify those which encode an active phytyl/prenyltransferase, ..."

While claims 31, 33, 34, and 36 do recite the use of various sequences that upon expression modulate phytyl/prenyltransferase or tocopherol levels, this factor does not contribute to a lack of enablement. In fact, in contrast to the view of the Examiner, the specification provides sufficient guidance for one skilled in the art to make and use the claimed invention.

The Examiner asserts the specification does not provide sufficient guidance to make and use the sequences recited in claims 31, 33, 34 and 36. This assumption is incorrect. As discussed above, phytyl/prenyltransferase polynucleotides and their conserved regions were known in the art at the time of filing of the present invention (Lopez et al., 1996). Guidance has been provided throughout the specification for determining percent sequence homology, and stringent hybridization conditions (see the specification; page 6, beginning on line 10 for sequence homology and page 12, beginning on line 30 for hybridization). Generating such sequences are routine.

Descriptions of phenotypic or enzymatic changes resulting from modulated phytyl/prenyltransferase protein levels can be found in the specification on page 33, 6-16 (describing lack of tocopherols in the phytyl/prenyltransferase knock-out mutant ΔSLR 1736); page 40, lines 21-34 and page 41, lines 1-10 (describing confirmation of prenyltransferase activity); page 58, lines 29-34 and page 59, full page (describing modulation of tocopherol levels in soybean somatic embryos containing the transgene represented by SEQ ID NO: 3).

Thus, a rational scheme for identifying sequences encompassed by claims 31, 33, 34 and 36 has been provided in the specification. The skilled artisan could choose among possible modifications to produce sequences within the parameters

of the claims and test these modified variants to determine if, upon expression, phytyl/prenyltransferase activity is increased or decreased.

The Examiner is reminded that the Federal Circuit has repeatedly stated that enablement is not precluded by the necessity for some experimentation, so long as the experimentation needed to practice the invention is not undue. *In re Wands* 8 USPQ2d 1400 (Fed. Cir., 1988). Furthermore, a considerable amount of experimentation is permissible, if it is merely routine, or if the specification provides a reasonable amount of guidance in which the experimentation should proceed.

In the instant case, the quantity of experimentation required to identify sequences encompassed by claims 31, 33, 34 and 36, amounts to two steps: generating a nucleotide sequence encompassed by the claims and assaying for modulated activity. As discussed above, such assays are not only routine in the art, but have further been presented in the specification.

Ample guidance is therefore provided to allow one of skill in the art identify additional sequences encompassed by claims 31, 33, 34 and 36. Consequently, the amount of guidance presented in the specification is sufficient to enable the claimed methods recited in claims 31, 33, 34 and 36.

The Examiner concludes: "The state of the art for modification of gene expression or of phenotypic characteristics in plants by genetic transformation is highly unpredictable and hence significant guidance is required to practice the art without undue experimentation .... Given the unpredictability in the art of plant transformation to obtain a specified phenotype, the instant invention is not enabled given the lack of guidance in the specification with regard to what nucleic acids other than SLR1736 can be expected to result in a modulation of phytyl/prenyltransferase levels or tocopherol levels ...."

While the claimed methods require selection of transformed plants exhibiting the desired traits/phenotype, the selection is routine and would not require undue experimentation. As discussed above, assays for detecting the phytyl/prenyltransferase modulated phenotype are provided in the present

specification. No matter how much detail is provided, one will have to select for the desired phenotype.

The Examiner's allegation regarding undue experimentation does not properly recognize the routine nature of such screening. Screening a large number of transformants to obtain a desired phenotype is not only routine, but is the very heart of the biotechnological arts. It is the very provision of such screening assays that enables one of skill in the art to select transformants with modified phytyl/prenyltransferase function as described in the specification.

It is believed that the specification has provided sufficient disclosure and enablement so that one skilled in the art could readily make the embodiments encompassed by the presently claimed invention.

In view of the above arguments and amendments, it is respectfully requested that the rejection of claims 22-39 under 35 U.S.C. §112, first paragraph, be withdrawn.

Rejections under 35 USC §102

Claims 22, 24, and 25 are rejected under 35 USC §102(b) as being anticipated by Ausich et al.

The Examiner states: "Ausich et al. teach a method for modulating the level of geranylgeranyl pyrophosphate (GGPP) synthase (which is a phytyl/prenyltransferase protein) in a plant ...."

The MPEP states: "... for anticipation under 35 USC §102, the reference must teach every aspect of the claimed invention either explicitly or impliedly. Any feature not directly taught must be inherently present" (MPEP 706.02).

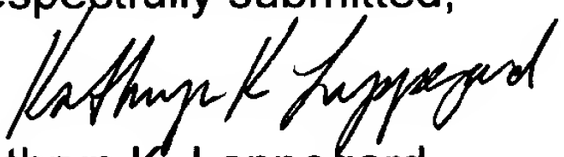
The Examiner asserts that the geranylgeranyl pyrophosphate synthase is a phytl/prenyltransferase. Not only is this assertion not supported by the art as discussed herein under §112, but a cursory examination of the respective names of the proteins indicate that the functions of the proteins are different. Quite simply, a synthase does not function as a transferase.

The reference cited by the Examiner does not teach any of the features of the presently claimed invention and thus does not anticipate. It is respectfully requested that the rejections under 35 USC §102 be withdrawn.

CONCLUSION

On the basis of the above amendments and remarks, reconsideration of the application and its allowance are respectfully requested.

Respectfully submitted,



Kathryn K. Lappegard
Agent for Applicant(s)
Registration No. 46,857

PIONEER HI-BRED INTERNATIONAL, INC.
Corporate Intellectual Property
7100 N.W. 62nd Avenue
P.O. Box 1000
Johnston, Iowa 50131-1000
Phone: (515) 253-5707
Facsimile: (515) 334-6883

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification:

Paragraph beginning at line 10 of page 6 has been amended as follows:

Methods of alignment of sequences for comparison are well-known in the art. Two methods are used herein to define the present invention. The first is the BLAST 2.0 suite of programs using default parameters. Altschul et al., *Nucleic Acids Res.* 25:3389-3402 (1997). Software for performing BLAST analyses is publicly available, e.g., through the National Center for Biotechnology Information [(http://www.ncbi.nlm.nih.gov/)]. The second is the GAP program, available as part of the Wisconsin Genetics Software Package, that uses the algorithm of Needleman and Wunsch (*J. Mol. Biol.* 48:443-453, 1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. Default gap creation penalty values and gap extension penalty values in Version 10 of the Wisconsin Genetics Software Package for nucleotide sequences are 50 and 3, respectively, and for protein sequences are 8 and 2, respectively. Unless otherwise specified, references to the GAP program or algorithm refer to the GAP program or algorithm in version 10 of the Wisconsin Genetics Software Package. The gap creation and gap extension penalties can be expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package for nucleotide sequences are 50 and 3, respectively, and for protein sequences are 8 and 2, respectively. Unless otherwise specified, references to the GAP program or algorithm refer to the GAP program or algorithm in version 10 of the Wisconsin Genetics Software Package. The gap creation and gap extension penalties can be

expressed as an integer selected from the group of integers consisting of from 0 to 200. Thus, for example, the gap creation and gap extension penalties can be 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65 or greater. The scoring matrix used in Version 10 of the Wisconsin Genetics Software Package is BLOSUM62 (*see* Henikoff & Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915).

Paragraph beginning at line 6 of page 32 has been amended as follows:

PCC 6803 was used as a tool for identification of genes encoding enzymes involved in biosynthesis of tocopherols. *Synechocystis* is a cyanobacterium capable of tocopherol biosynthesis. The entire genome of this photosynthetic organism has been recently sequenced (Kaneko et al., 1996) and the data is available on a public searchable database, called CyanoBase [(http://www.kazusa.or.jp/cyano/cyano.html)]. Using CyanoBase, we have identified an open reading frame (SLR1736) encoding a phytyl/prenyltransferase involved in the biosynthesis of 2-methyl-6-phytylplastoquinol, one of the tocopherol precursors. This open reading frame was identified based on similarity with the phytyl/prenyltransferase SLR0056, a phytyl/prenyltransferase involved in the biosynthesis of chlorophyll in *Synechocystis* PCC 6803. SLR0056 exhibits a high homology with the previously identified chlorophyllide/phytyl/prenyltransferases from many cyanobacteria and *A. thaliana* (Lopez et al., 1996), suggesting that this enzyme is also involved in chlorophyll synthesis.

Paragraph beginning at line 28 of page 33 has been amended as follows:

Chromosomal DNA from wild type Synechocystis PCC 6803 was isolated according to Williams (Methods in Enzymology (1987) 167:766-778). The [following] primers <u>represented by SEQ ID NOS: 7 (SLR1736F) and 8 (SLR1736R)</u> were

designed using Mac Vector computer program to amplify a 1.022 kb fragment containing the SLR1736 open reading frame[:].

[SLR1736F: 5'-TATTCATATGGCAACTATCCAAGCTTTTTG-3'
SLR1736R: 5'-GGATCCTAATTGAAGAAGATACTAAATAGTTC-3']

NdeI and BamHI sites were added to the primers to facilitate sub-cloning for expression purposes. ATG in the SLR1736F primer is the start codon for the SLR1736 open reading frame published in the CyanoBase Web-site. Taq polymerase (Gibco BRL) was used for gene disruption purposes and later Vent polymerase (NEB) was used for expression purposes following the manufacturer's recommendations. The following cycles were performed:
For Taq polymerase amplification:
95°C/5 minutes (1 cycle)
95°C/45 seconds, 45°C/45 seconds, 68°C/45 seconds (5 cycles)
95°C/45 seconds, 52°C/45 seconds, 72°C/45 seconds (30 cycles)
72°C/10 minutes
The same thermocycler conditions were used to amplify SLR1736 with Vent polymerase except that elongation times were extended to 2 minutes.

Paragraph beginning at line 15 of page 39 has been amended as follows:

A developing seed-specific cDNA library from A.thaliana (lambda-ZAP type, provided by John Ohlrogge at the Michigan State University) was screened using a PCR product from wild type A. thaliana genomic DNA (Ler ecotype) which exhibits a high degree of homology with the Synechocystis phytyl transferase. Primers represented by SEQ ID NO: 5 (AT1736F) [5'-TTGTTTTCAGGCTGTTGTTGCAGCTCTC-3')] and SEQ ID NO: 6 (AT1736B) [(5'-CGTTTCTGACCCAGAGTTACAGAGAATG-3')] were used to amplify about 1kb

fragment corresponding to 60238 – 61229 bp region of the BAC clone F19F24 (A. thaliana database at Stanford). The following program was used to amplify this fragment with Vent DNA polymerase (New England Biolabs):

95°C/5 minutes (1 cycle)

95°C/45 seconds; 50°C/45 seconds, 72°C/1 minute (30 cycles)

72°C/10 minutes (1 cycle).

Paragraph beginning at line 27 of page 39 (final paragraph) has been amended as follows:

The PCR product was then sub-cloned into *Eco*RV site of pBluescript KS (Stratagene) as in the case of the cyanobacterial phytyl transferase presented above and sequenced from both ends using T3 and T7 primers (Stratagene) to ensure the identity of the sub-cloned fragment. A 300 bp fragment of the insert (5'-end) was released with *Eco*RI from the vector and used as a radioactively-labeled probe to obtain full-length clones. About 2.5 million plaques of the seed-specific library were screened using standard procedures (Sambrook, J.; Fritsch, E.F. & Maniatis, T. (1989). Molecular Cloning. 2nd edition, Cold Spring Harbor Laboratory Press). 16 positive non-purified plaques were chosen for PCR analysis using T3 and SEQ ID NO: 30 (AT1736T7c) [(5'-GACATATTTTTGCAGTCTGCC-3)] which is an internal primer for the phytyl transferase. Clones #1, 3, 5, 8, 11, 12, and 14 were selected for further purification and single clone excision, performed according to manufacturer (Stratagene), to obtain individual clones in pBluescript SK plasmids. Each clone was sequenced from each end using T3 and T7 primer. The longest clone, #11 – about 1.6 kb, was chosen for complete sequencing which is in progress now. All clones were aligned to the genomic clone F19F24 from *A. thaliana* to confirm their identity, identify introns and find possible sequencing mistakes in the genomic sequence. We believe that ATG codon (59220 bp on F19F24) is the start codon of the phytyl transferase involved in tocopherol synthesis in *A. thaliana*.

Starting from this methionine, the first 36 amino acids represent the chloroplast thylakoid membrane-targeting sequence [(PSORT program, hhtp://psort.nibb.ac.jp:8800/)].

Paragraph beginning at line 25 of page 43 (final paragraph) has been amended as follows:

Colony hybridization is conducted as described by Sambrook, J., Fritsch, E.F. and Maniatis, T., (in Molecular Cloning: A laboratory Manual, 2nd Edition). The following probes were used in colony hybridization:

1. First strand cDNA from the same tissue as the library was made to remove the most redundant clones.
2. 48-192 most redundant cDNA clones from the same library based on previous sequencing data.
3. 192 most redundant cDNA clones in the entire corn sequence database.
4. A Sal-A20 oligo nucleotide[: TCG ACC CAC GCG TCC GAA AAA AAA AAA AAA AAA AAA,] removes clones containing a poly A tail but no cDNA.
5. cDNA clones derived from rRNA.

Paragraph beginning at line 8 of page 44 has been amended as follows:

Gene identities were determined by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., (1993) *J. Mol. Biol.* 215:403-410; [see also www.ncbi.nlm.nih.gov/BLAST/)] searches under default parameters for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences were analyzed for similarity to all publicly available DNA sequences contained in the

"nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J. *Nature Genetics* 3:266-272 (1993)) provided by the NCBI. In some cases, the sequencing data from two or more clones containing overlapping segments of DNA were used to construct contiguous DNA sequences.

Paragraph beginning at line 15 of page 55 has been amended as follows:

The ability to change the levels of total tocopherol levels in plants by transforming them with sequences encoding the maize phytyl/prenyltransferase was tested by preparing transgenic soybean somatic embryos and assaying the tocopherol and oil levels. Plasmid DNA from clone poo18chste82r was used as a template for the amplification of the open reading from pcr by using the [following two] primers [AGC GCG GCC GCA TGG ACG CGC TTC GCC TAC GGC CGT] represented by SEQ ID NO: 31 (forward primer) and [AGC GCG GCC GCT CAC CGC ACC AGA GGG ATG AGC AG] SEQ ID NO: 32 (reverse primer). Pfu polymerase was used according to the manufacturers recommendations (Stratagene). The following pcr reaction mix contained the following: 5ng plasmid, 25nmoles dNTPs, 5% DMSO, 1x pcr buffer (supplied), 30nmoles primers, 5U pfu polymerase in 100ul reaction volume. The pcr reaction conditions were as follows Step 1, 45s 94°C; step 2 25 cycles of 45s 94°C, 45s 58°C[c] annealing, 2min extension 72°C. Step 3 72°C 10min, step 4 0°C. The pcr product was purified by agarose gel electrophoresis (!% agarose in TAE), the ethidium bromide visualized band cut out and purified from the gel by using a QIAquick Gel Extraction kit (Qiagen) according to the manufacturers recommendations. The purified pcr product (200ng) was ligated into the srf I site of the plasmid PCR-Script cloning vector and the resultant plasmid was used to transform E.coli DH10 cells. Colonies

containing the 1.2kb NotI fragment were identified by antibiotic (ampicillin selection) and blue / white (IPTG + X-gal) selection of colonies on LB/Amp plates. White (recombinant) colonies were picked and grown overnight on liquid LB/Amp culture. Positive clones were identified by plasmid preparation and restriction digest analysis for the presence of the 1.2kB NotI fragment. Positive clones were used as template to fully sequence the phytyl transferase of (both strands). Plasmids containing the correct insert verified by nucleic acid sequence were digested with NotI and the 1.2kb fragment ligated to NotI-digested and phosphatase-treated pKS67. The plasmid pKS67 was prepared by replacing in pRB20 (described in U.S. Patent No. 5,846,784) the 800 bp Nos 3' fragment, with the 285 bp Nos 3' fragment containing the polyadenylation signal sequence and described in Depicker et al. (1982) *J. Mol. Appl. Genet.* 1:561-573. Clones were screened for the sense and antisense orientation of the phytyl/prenyltransferase insert fragment by restriction enzyme digestion.

In the Claims:

Claims 1-21- 29, 30, 37, 38, and 39 have been cancelled without prejudice.

Claims 22, 23, 24, 27, 28, 31, 32, 33, 34, 35, and 36 have been amended as follows:

22. (Amended) A method for modulating the level of phytyl/prenyltransferase protein in a plant, comprising:

(a) stably transforming a plant cell with a phytyl/prenyltransferase polynucleotide operably linked to a promoter, wherein the polynucleotide is in sense or antisense orientation;

(b) growing the plant cell under plant growing conditions to produce a regenerated plant which expresses [capable of expressing] the

polynucleotide for a time sufficient to modulate the level of phytyl/prenyltransferase protein in the plant.

23. (Amended) The method of claim 22, wherein the phytyl/prenyltransferase polynucleotide is [selected from those of] SEQ ID NO: [1,] 3, [9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29].

24. (Amended) The method of claim 22, wherein the plant is corn, soybean, sunflower, sorghum, canola, wheat, alfalfa, cotton, rice, barley, millet, [or] *Arabidopsis thaliana*, tomato, *Brassica,* pepper, potato, apple, [vegetables, peppers, potatoes, apples,] spinach, or lettuce.

27. (Amended) A method for modulating the level of tocopherol in a plant, comprising:

(a) stably transforming a plant cell with a phytyl/prenyltransferase polynucleotide operably linked to a promoter, wherein the polynucleotide is in sense or antisense orientation;

(b) growing the plant cell under plant growing conditions to produce a regenerated plant which expresses [capable of expressing] the polynucleotide for a time sufficient to modulate level of tocopherol in the plant.

28. (Amended) The method of claim 27, wherein the phytyl/prenyltransferase polynucleotide is [selected from] SEQ ID NO: [1,] 3[, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29].

31. (Amended) The method of claim 22, wherein the phytyl/prenyltransferase polynucleotide comprises a member selected from the group consisting of:

(a) a polynucleotide having at least 70% sequence identity to the entire coding sequence of SEQ ID NO [Nos.]: [1,] 3, [9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29,] wherein the % sequence identity is determined by GAP using default parameters, and

(b) a polynucleotide complimentary to a polynucleotide of (a).

32. (Amended) The method of claim 22, wherein the phytyl/prenyltransferase polynucleotide comprises a member selected from the group consisting of:

(a) a polynucleotide that encodes a polypeptide of SEQ ID NO [Nos.]: 4[, 12, 14, 16, 18, 20, 22, 24, 26 or 28];

(b) a polynucleotide comprising the sequence set forth in SEQ ID NO [Nos.]: 3[, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29]; and

(c) a polynucleotide complementary to a polynucleotide of (a) or (b).

33. (Amended) The method of claim 22, wherein the phytyl/prenyltransferase polynucleotide comprises a polynucleotide which selectively hybridizes, under stringent hybridization conditions and a wash in 2X SSC at 50°C, to a hybridization probe the polynucleotide sequence of which consists of the coding sequence of SEQ ID NO [Nos.]: 3[, 11, 13, 15, 17, 19, 21, 23, 25 or 27, or the complement of the coding sequence of SEQ ID Nos.: 3, 11, 13, 15, 17, 19, 21, 23, 25 or 27].

34. (Amended) The method of claim 27, wherein the phytyl/prenyltransferase polynucleotide comprises a member selected from the group consisting of:

(a) a polynucleotide having at least 70% sequence identity to the entire coding sequence of SEQ ID NO [Nos.]: [1,] 3[, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29], wherein the % sequence identity is determined by GAP using default parameters, and

(b) a polynucleotide complimentary to a polynucleotide of (a).

35. (Amended) The method of claim 27, wherein the phytyl/prenyltransferase polynucleotide comprises a member selected from the group consisting of:

(a) a polynucleotide that encodes a polypeptide of SEQ ID NO [Nos.]: 4[, 12, 14, 16, 18, 20, 22, 24, 26 or 28];

(b) a polynucleotide comprising the sequence set forth in SEQ ID NO [Nos.]: 3[, 11, 13, 15, 17, 19, 21, 23, 25, 27 or 29]; and

(c) a polynucleotide complementary to a polynucleotide of (a) or (b).

36. (Amended) The method of claim 27, wherein the phytyl/prenyltransferase polynucleotide comprises a polynucleotide which selectively hybridizes, under stringent hybridization conditions and a wash in 2X SSC at 50°C, to a hybridization probe the polynucleotide sequence of which consists of the coding sequence of SEQ ID NO [Nos.]: 3, [11, 13, 15, 17, 19, 21, 23, 25 or 27,] or the complement of the coding sequence of SEQ ID NO [Nos.]: 3[, 11, 13, 15, 17, 19, 21, 23, 25 or 27].

into an exon or intron of an actively transcribed endogenous gene, thereby simultaneously disrupting the gene and acting as a locus-specific marker of the gene. This would greatly facilitate the identification and cloning of the disrupted genes, a task that is not necessarily straightforward following chemical mutagenesis. In practice, hundreds of transgenic embryos, each carrying multiple integration sites, could be generated per day and screened for GFP expression. A huge advantage of using this approach in frog embryos is that they develop externally, therefore GFP expression can be assayed in living embryos at any stage. Most embryos will not express the marker gene. These will be discarded and only the few that express will be nurtured to maturity, thus greatly reducing the number of embryos that must be carried to the next generation. Preliminary experiments in *X. laevis* strongly suggest that using a gene trap approach will be productive (O. Bronchain and E. Amaya, unpublished).

The powerful manipulations that one can perform on amphibian embryos have been used to reveal important principles about development for over a century. As we approach the next century, it appears that it will now be possible to overlay this rich embryological history with the power of genetic manipulations, creating an armamentarium of approaches as we look towards revealing a new generation of concepts about vertebrate embryonic development.

Acknowledgements

We are especially grateful to L. Zimmerman for his contributions to this project. This work was supported by NIH grant RR13221 to E.A. and R.M.G.

References

1 Hamburger, V. (1988) *The Heritage of Experimental Embryology: Hans Spemann and the Organizer*, Oxford University Press
2 Wu, M. and Gerhart, J. (1991) *Methods Cell Biol.* 36, 3–18
3 Bellerby, C.W. (1934) *Nature* 133, 494–495
4 Shapiro, H.A. and Zwarenstein, H. (1934) *Nature* 133, 762
5 Weisman, A.I. and Coates C.W. (1944) *The South African Frog (Xenopus laevis) in Pregnancy Diagnosis: A Research Bulletin*, New York Biologic Research Foundation
6 Bisbee, C.A. *et al.* (1977) *Science* 195, 785–787
7 Cannatella, D.C. and de Sa, R.O. (1993) *Syst. Biol.* 42, 476–507
8 Graf, J-D. and Kobel, H.R. (1991) *Methods Cell Biol.* 36, 19–34
9 Ohno, S. (1970) *Evolution by Gene Duplication*, Springer-Verlag
10 Tymowska, J. and Kobel, H.R. (1972) *Cytogenetics* 11, 270–278
11 Krotoski, D.M. *et al.* (1985) *J. Exp. Zool.* 233, 443–449
12 Gray, J.E. (1864) *Ann. Mag. Nat. Hist.* 14, 315–316
13 Cannatella, D.C. and Trueb, L. (1988) *Zool. J. Linn. Soc.* 94, 1–38
14 de Sa, R.O. and Hillis, D.M. (1990) *Mol. Biol. Evol.* 7, 365–376
15 Tymowska, J. (1973) *Cytogenet. Cell Genet.* 12, 297–304
16 Thiebaud, C.H. and Fischberg, M. (1977) *Chromosoma* 59, 253–257
17 Duellman, W.E. and Trueb, L. (1986) *Biology of Amphibians*, McGraw–Hill
18 Herskowitz, I. (1987) *Nature* 329, 219–222
19 Amaya, E. *et al.* (1991) *Cell* 66, 257–270
20 Kroll, K.K. and Amaya, E. (1996) *Development* 122, 3173–3183
21 Gossler, A. *et al.* (1989) *Science* 244, 463–465
22 Skarnes, W.C. *et al.* (1992) *Genes Dev.* 6, 903–918

Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression

MARY K. MONTGOMERY AND ANDREW FIRE
montgomery@macalester.edu • fire@mail1.ciwemb.edu

DEPARTMENT OF EMBRYOLOGY, CARNEGIE INSTITUTION OF WASHINGTON, 115 WEST UNIVERSITY PARKWAY, BALTIMORE, MD 21210, USA.

Many fundamental natural processes have been uncovered not by preplanned scientific enquiry, but serendipitously by engineers and scientists who observed unexpected consequences of their manipulations. Biologists routinely use engineering to manipulate the expression of specific genes and, thus, understand (or benefit from) their function. Sometimes we wish to make a particular gene silent; at other times we want the genes to 'talk' more loudly. Attempts at silencing have often employed an antisense strategy of introducing single-stranded nucleic acid from the noncoding strand to sequester or modify the native transcript, thereby preventing accumulation of the corresponding protein. Conversely, by introducing extra copies of a specific gene, one might expect in many cases to overproduce the corresponding mRNA and protein products. Although these techniques have been successful in numerous applications, a body of literature is emerging that documents certain cases in which unexpected outcomes of these manipulations are seen in organisms as diverse as nematodes and plants. These observations encompass 'transgene silencing' (a failure to express certain multicopy transgenes) and co-suppression (the ability of a 'sense' transgene to interfere with the activity of the endogenous genetic locus). Certain of these phenomena are thought to involve direct DNA–DNA interactions, whereas others have been proposed to require an RNA effector molecule. The structure and mechanistic properties of RNAs mediating the latter type of co-suppression have yet to be elucidated. Here, we discuss the possibility that double-stranded RNA (dsRNA), rather than sense or antisense single-stranded RNAs alone, is the effector molecule responsible for RNA-mediated silencing and co-suppression.

 0168-9525/98/$19.00
PII: S0168-9525(98)01510-8

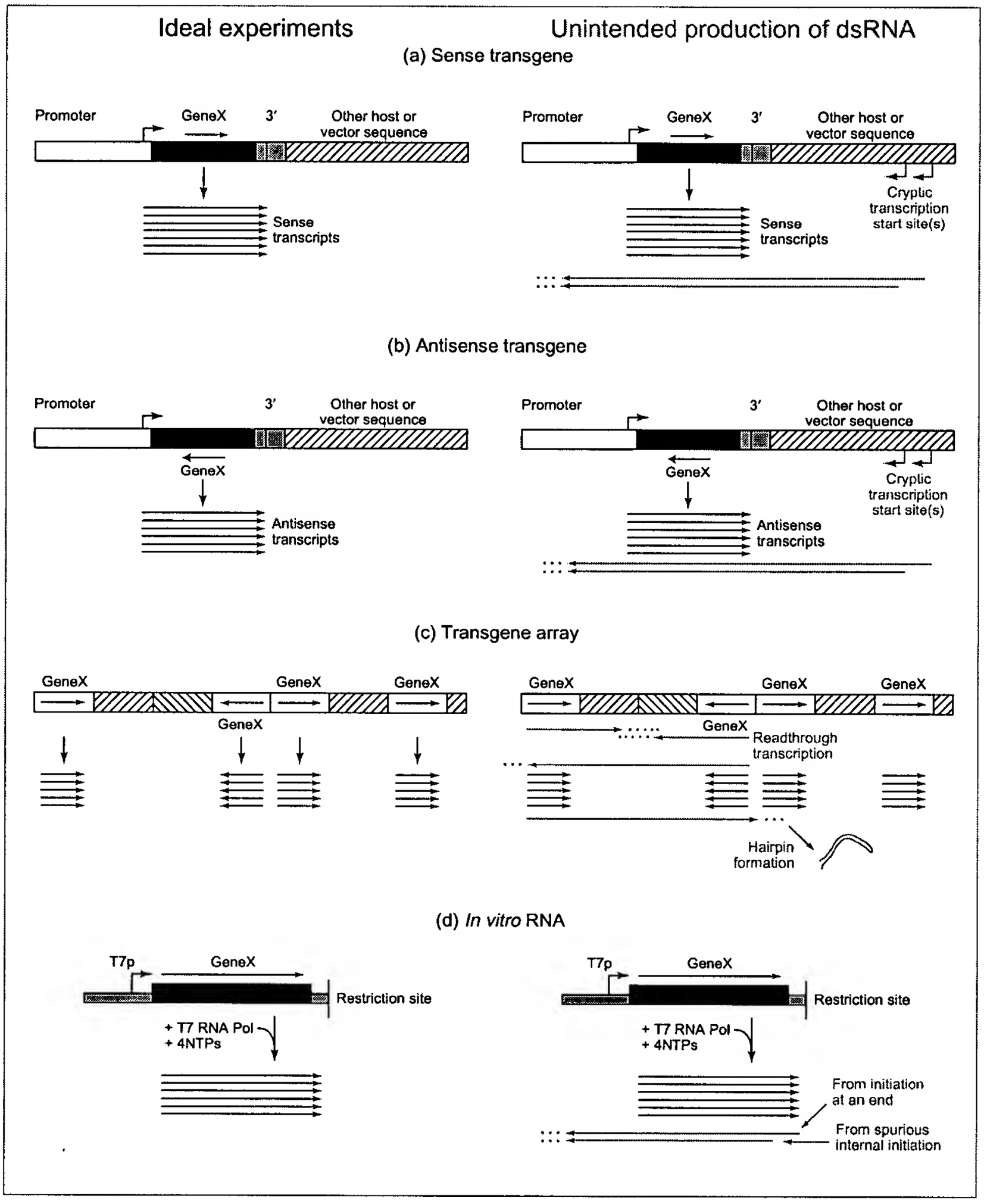


FIGURE 1. Unintended production of double-stranded (ds) RNA. Left, a series of theoretical experiments that might be designed to produce a pure population of single-stranded RNA. Right, how a low level of dsRNA could also be produced in each case. (a) A transgene designed to produce 'sense' RNA is transcribed at low level from a cryptic (or natural) start site on the opposite strand. Hybridization to 'sense' transcripts from the same template would result in dsRNA. (b) A transgene designed to produce 'antisense' could similarly be subject to low-level transcription on the opposite strand, with dsRNA resulting from hybridization of the newly transcribed RNAs. (Note that antisense RNA might alternatively hybridize with the endogenous chromosomal transcript to make dsRNA; it is not clear, however, that sense and antisense RNAs sythesized at distant nuclear sites would form dsRNA and be capable of interference.) (c) A transgene array containing tandem and inverted copies of a DNA construct ('geneX') might be expected to produce only one strand of RNA. Note, however, that readthrough of the geneX terminator would produce RNA with an inverted repeat structure. This RNA could undergo intramolecular hybridization to produce a predominantly double-stranded hairpin. (d) During *in vitro* synthesis of RNA, transcription initiates primarily at the bacteriophage RNA-polymerase promoter. Initiation can, however, also occur at internal sites and template ends, which leads to some inclusion of dsRNA in 'sense' and 'antisense' RNA preparations.

RNA-mediated genetic interference (RNAi) in the nematode *C. elegans*

Several years ago it was reported that antisense RNA targeted to specific endogenous genes in *Caenorhabditis elegans*, when either expressed from a transgene[1] or injected directly into the worm's gonad[2], could phenocopy a null or hypomorphic mutation in the targeted gene. Surprisingly, both reports indicated that sense transcripts also were effective in producing the targeted phenotype. These observations were difficult to explain using a conventional model of antisense sequences inactivating the sense transcript. Recently, at least part of this mystery was solved by the discovery that much, if not all, of the genetic interference from injected 'sense' and 'antisense' RNA is actually mediated by double-stranded RNA (dsRNA) that is present at a low level in all *in vitro* RNA syntheses because of the non-specific activity of RNA polymerases[3] (Fig. 1). Highly purified preparations of antisense (and sense) RNAs had negligible effects, whereas dsRNA exhibited potent and specific interference against any of a variety of targeted genes. The potency of RNAi in worms points to the existence of novel mechanism(s)[3]. Even with an abundantly transcribed target (several thousand mRNA copies per cell), a few molecules of dsRNA per cell can produce specific inhibition. This would not be expected from a simple antisense mechanism; there is simply not enough material to bind to all the endogenous mRNA targeted for destruction. These results generated two fundamental questions: how can dsRNA mediate gene-specific interference; and what is the physiological purpose of this process?

RNA-mediated silencing and co-suppression in plants

A second body of work on RNA-mediated interference comes from the plant world[4]. In the late 1980s, plant researchers were surprised to find that the introduction of certain transgenes into plants can result in homology-dependent silencing of an endogenous locus (rather than overexpression of the coding region of interest). This phenomenon is referred to as co-suppression. Not all transgenes cause this effect; there is no current basis for predicting which would and which would not. Gene silencing in plants has been proposed to encompass a variety of different mechanisms[4,5], including some that act by direct DNA–DNA interaction and others that involve interference by an RNA product of the transgene. Strong evidence for the latter class of mechanisms comes from experiments in which RNAs are introduced in the absence of a DNA template (using RNA viruses as vectors); the ability of viral RNAs to interfere with a homologous gene in the plant genome is one of the strongest arguments for the existence of RNA-mediated silencing mechanisms[6–8].

The literature contains a few clues as to the nature of the interfering RNA. In certain cases, co-suppression is correlated with high-level transcription of the transgene[9,10]. Given recent results in *C. elegans*, we pose the possibility that transcription along the antisense strand of a transgene could result in low levels of interfering dsRNA (Fig. 1). Such transcription might be low-level synthesis directed by sequences within the vector or flanking regions at the site of integration (e.g. see Ref. 11). Significantly, Que *et al.*[10] reported that, whereas co-suppression was associated with accumulation of transcripts at high concentrations from single-copy transgenes, inversely repeated transgenes could cause co-suppression, irrespective of promoter strength or level of the transgene mRNA. Transcripts from inversely repeated transgenes would be expected to produce a double-stranded structure. Experiments with chimeric RNA viruses[7,8] might similarly point toward a dsRNA involvement; in these experiments, the viral RNA replicase copies the chimeric RNA in the cytoplasm, generating both sense and antisense material.

Similarities between nematodes and plants

RNAi in worms and co-suppression in plants share some striking similarities. Both are cases of gene-specific interference. dsRNA has been shown to be the agent of interference in nematodes and, as suggested above, there is some indication that dsRNA could also be responsible for co-suppression in plants. Perhaps the most interesting common characteristic is that the phenomenon can spread from the site of interfering RNA synthesis or application. In worms, the dsRNA mix can be injected into the body cavity, where it can produce an interfering effect in distant tissues and in F1 progeny, indicating that cells may have an RNA-transport mechanism[3]. Similarly, two groups of researchers have demonstrated the systemic spread of co-suppression in plants[12,13]. An RNA molecule, spreading throughout the plant via phloem, has been proposed as the mobile agent responsible for transmitting the co-suppression state[12].

Possible mechanisms for RNA-mediated interference

The sub-stoichiometric activity of the interfering RNA in *C. elegans* led to various models: that interference involves a catalytic mechanism dependent on the injected RNA; that the input material is amplified; or that interference occurs at the level of the gene. Several lines of evidence argue against DNA in the genome as a target for RNAi. Effects of dsRNA are generally not heritable beyond the first generation; injected animals and progeny exhibit the effects of RNAi, whereas animals of the F2 generation generally revert to a wildtype phenotype[3]. Additional evidence comes from direct sequencing of genomic DNA following RNA-mediated interference with *unc-22*; these experiments yielded no indication of mutations in the target gene (S. Xu and A. Fire, unpublished). Consistent with an RNA target, interference was effective using a variety of regions present in mature RNA, but was not effective using intronic or promoter sequences[3]. At this point, one attractive hypothesis is that dsRNA might result in early degradation of the endogenous mRNA. We know from *in situ* hybridization studies that RNA transcripts of a target gene fail to accumulate after RNAi (Ref. 3). Conceivably, the lack of mRNA products could be an indirect consequence of blocked processing or transport. Alternatively, endogenous transcripts could be degraded by a sequence-specific mechanism directed by dsRNA.

The mechanisms mediating certain co-suppression phenomena in plants have been shown to act both on the DNA template and on RNA products. Wassenegger *et al.*[6] showed that viral or transgene-generated RNA could direct *de novo* modification (presumably methylation) of a homologous sequence in the plant genome. Other studies provide cases in which RNA-mediated co-suppression acts

post-transcriptionally, potentially by the rapid degradation of the target transcript[14]. In one example, transgene-mediated silencing of the endogenous gene encoding β-1,3-glucanase in tobacco, de Carvalho Niebel and colleagues[15] demonstrated that the suppressed genes are actively transcribed. Subsequently, Jacobs *et al.*[16] showed that gene silencing in this line correlates with an increased turnover of both the transgenic and the endogenous transcripts of β-1,3-glucanase. In the case of virally provided RNA sequences, it appears that viral RNA molecules can serve as targets as well as 'triggers' for co-suppression[8,13,17]. Could there be a mechanistic link between (a) RNA-mediated degradation of RNA and (b) RNA-mediated methylation of DNA? One possibility is that these are two separate processes mediated by similar RNA molecules; alternatively, there could be a causal relationship, perhaps from an ability of RNA decay products to trigger methylation of homologous sequences in replicating DNA.

Does RNA-mediated interference do a job for the cell?

In addition to the mechanistic questions, attention is also merited to the physiological role for the RNA-associated silencing phenomenon. A role for co-suppression mechanisms in systemic defense against viruses has been suggested for plants[17,18] and could apply to other organisms as well. Such a response represents an effective means by which to prevent viral replication and induce resistance in surrounding tissues prior to viral invasion.

Alternatively, co-suppression/RNAi might modulate normal gene expression. One can easily imagine double-stranded RNAs being used by the cell as a potent means to turn off specific genes in response to physiological or developmental cues. Perhaps the best way to identify these processes will be to find mutants that are defective in carrying out RNAi.

Do RNA-interference mechanisms have counterparts outside of plants and nematodes?

Mammalian cells exhibit a global antiviral response to double-stranded RNA. In this response, the PKR protein kinase recognizes dsRNA and unleashes a vehement but somewhat non-specific response leading to general translational arrest[19]. Intriguingly, this type of systemic response can occur if the dsRNA is provided extracellularly[20] (consistent with the possibility of dsRNA uptake by mammalian cells). Viruses have evolved a number of strategies for evading or inhibiting the PKR response[21]. Certain tissue-culture cell lines lack PKR and are susceptible to mutant viruses that would otherwise be non-virulent. Any gene-specific interference by dsRNA in PKR-proficient mammalian cells would be dependent on a transient lapse in the PKR response, or on a controlled level of dsRNA that was incapable of activating PKR.

A wealth of information indicates that specific RNA-mediated interference mechanisms contribute to the control of gene expression in vertebrate and other systems. For many of these contributions, the precise nature of the interfering RNA (single-stranded versus double-stranded material) has yet to be characterized. Antisense transcripts have been reported for large numbers of vertebrate genes[22]. In some cases, roles for these transcripts in regulating the sense transcripts from the opposite strand have been demonstrated. From an informatics perspective, a surprisingly large fraction of vertebrate mRNAs contain long-conserved sequences within the 3′ untranslated region as well as long blocks without silent changes in their protein-coding regions[22]. Lipman[22] has proposed that these conserved sites are regulatory targets of endogenous antisense transcripts encoded by the complementary strand of the gene. Such a mechanism would, thus, be common and relatively conserved. Endogenous genes regulated by antisense transcripts have also been described for the primitive eukaryote *Dictyostelium*, and such mechanisms have been studied in detail in Eubacteria and Archaebacteria (reviewed in Ref. 23). Co-suppression phenomena, similar to that described for plants, have also been observed in *Dictyostelium*[24]. It will be interesting in the next few years to learn whether any or all of these effects share underlying mechanistic features and we suggest, moreover, that by studying the mechanisms underlying these phenomena, we will be better able to interpret the native language of the cell.

Acknowledgements

We thank J. Hsieh, D. Lipman, S. Parrish, M. Singer, S. Xu, members of our laboratory and an anonymous reviewer for helpful suggestions. Work in this laboratory is supported by grants R01-GM37706 and F32-GM17164 from NIGMS and by the Carnegie Institution of Washington.

References

1 Fire, A. *et al.* (1991) *Development* 113, 503–514
2 Guo, S. and Kemphues, K. (1995) *Cell* 81, 611–620
3 Fire, A. *et al.* (1998) *Nature* 391, 806–811
4 Matzke, M. and Matzke, A. (1995) *Plant Physiol.* 107, 679–685
5 Baulcombe, D.C. (1996) *Plant Mol. Biol.* 32, 79–88
6 Wassenegger, M. *et al.* (1994) *Cell* 76, 567–576
7 Kumagai, M.H. *et al.* (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 1679–1683
8 Angell, S.M. and Baulcombe, D.C. (1997) *EMBO J.* 16, 3675–3684
9 English, J.J. *et al.* (1997) *Plant J.* 12, 597–603
10 Que, Q. *et al.* (1997) *Plant Cell* 9, 1357–1368
11 Grierson, D. *et al.* (1991) *Trends Biotechnol.* 9, 122–123
12 Palauqui, J.C. *et al.* (1997) *EMBO J.* 16, 4738–4745
13 Voinnet, O. and Baulcombe, D.C. (1997) *Nature* 389, 553–553
14 Van Blokland, R. *et al.* (1994) *Plant J.* 6, 861–877
15 deCarvalho-Niebel, F. *et al.* (1995) *Plant Cell* 7, 347–358
16 Jacobs, J.J.M.R. *et al.* (1997) *Plant J.* 12, 885–893
17 Jorgensen, R.A. *et al.* (1998) *Science* 279, 1486–1487
18 Ratcliff, F., Harrison, B. and Baulcombe, D. (1997) *Science* 276, 1558–1560
19 Proud, C. (1995) *Trends Biochem. Sci.* 20, 241–246
20 Absher, M., and Stinebring, W.R. (1969) *Nature* 223, 715–717
21 Mathews, M.B. (1995) *Curr. Top. Microbiol. Immunol.* 199, 173–187
22 Lipman, D.J. (1997) *Nucleic Acids Res.* 25, 3580–3583
23 Nellen, W. and Lichtenstein, C. (1993) *Trends Biochem. Sci.* 18, 419–423
24 Scherczinger, C.A. and Knecht, D.A. (1993) *Antisense Res. Dev.* 3, 207–217